# Application of a novel enzymatic sterilisation Time Temperature Integrator

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## Abstract

A highly stable amylase has been identified from a hyper-thermophilic archaeon *Pyrocoocus furiosus*, which is suitable for the construction of Time Temperature Integrators (TTIs) for use in the food industry. Previously, amylases from terrestrial *Bacilli* have provided the feed stock for enzymatic TTIs. However, their application has been limited to pasteurisation; the new amylase now makes possible the application of such technology to sterilisation processes. The amylase demonstrates excellent thermal properties for its application as a "safety" TTI; namely an isothermal decimal reduction time at $121.1^{o}C$ of 22.5 ±4 minutes (21.5 ±2 under non isothermal) and a z-value of 10 ±1.5 $^{o}C$.

The enzymatic TTI have a distinct number of advantages over other temperature measurement technologies, e.g. thermocouples or data loggers, for the examination of continuous processes. Their small and near neutrally buoyant construction, 2.5mm x 8.0mm with 25µl of enzyme solution, makes them ideal for introduction into fluids or within food particulates. They are robust enough to pass through nearly all processing equipment; the encapsulation is from silicon tubing with elastomer plugs which also has thermal properties close to that of water. Post processing analysis is quick, inexpensive and accurate also the enzyme, an amylase is generally regarded as safe.

The sterilisation TTIs have been tested in reel & spiral and Largarde steam air retorts in cans and doypacks and showed $F_0$-values appropriate to the log reductions of *Clostridium botulinum* spores.

## Introduction

Thermal processing is probably the most important method for preserving food, and sterilisation processes such as canning are still immense business. The most important heat-resistant pathogen that might survive the thermal processing of low-acid foods is the spore-forming organism *Clostridium botulinum;* a sterilisation process must reduce the probability of a single *Clostridium botulinum* spore surviving in a low-acid product to one in $10^{12}$ the so called `botulinum cook'. This is the standard process and is achieved by an equivalent process of 3 minutes at 121.1°C, referred to as Fo 3 (FDA, 2005).

Additionally, manufacturers must prove that their products and processes are

safe. Validation is usually carried out with thermocouple or loggers, but this can be difficult for products with freely moving particulates or for some packaging types. If temperature probes cannot be used, alternative approaches to validating microbiological process safety are required, such as:

- Microbiological methods, where non-pathogenic analogue organisms, with similar temperature-induced death kinetics to the target pathogen are used (Brown, *et al.,* 1984). The enumeration of the post process surviving organisms allows for the calculation of the log reduction and sterilisation value.

- Simulated trials carried out in a laboratory where the heat transfer conditions of the process are replicated process value estimates are then made via models such as Ball (1923), NumeriCAL (FMC Inc., USA) or CTemp (Tucker, *et al.,* 1996) predict the required process conditions to achieve a desired sterilisation value.

- Mathematical process models that predict, for example, the temperature-time history of the critical food during a process (Sastry, 1986; McKenna and Tucker, 1991).

Another method that has extensively been applied to pasteurisation processes are time-temperature integrators (TTIs). A TTI can be defined as a small measuring device that shows a time-temperature dependent irreversible change that mimics the change of a target attribute when exposed to the same conditions. In practice, a TTI can be an enzyme, such as amylase or peroxidase, that denatures as it is heated in a buffer. If the reaction kinetics of the temperature-induced denaturation match those of the first order microbial death kinetics, the enzyme can be used as a biochemical marker of a process. The development of TTIs has received considerable attention recently (see reviews by Maesmans *et al.,* 1994; Hendrickx *et al.,*1995). It is now possible to use an amylase-based TTI for most commercial pasteurisation processes, from 70°C up to 95°C. Usually, TTIs use amylases from bacterial sources such as *Bacillus amyloliquefaciens* or *licheniformis* and the feasibility of extending its useable range upwards into sterilisation temperatures was demonstrated by drying amylases to precise moisture levels (Van Loey, *et al.,* 1997, Guiavarc'h, 2003). Laboratory results were encouraging and showed that different levels of moisture content gave a range of heat stabilities. However, practical issues made industrial application difficult (Tucker and Wolf, 2003). Therefore, a different method is required for a sterilisation TTI.

Also Tucker *et al.* (2005) specifically, the measured z-values for different amylases and found them to be in the range 9 to 10°C, ideal for bacterial spore destruction. Hence, an amylase was considered to provide the greatest chance of finding a TTI for use in sterilisation processes. The key was to locate an organism that has evolved in high temperature conditions and that produces amylase as it metabolises. Microorganisms from hostile environments such as volcanic vents seemed attractive (Stetter, 1996). These `hyperthermophilic' organisms represent a relatively unexplored research area for food applications but one with enormous potential for supply of heat stable enzymes; as a number of bacteria capable of growing at or above 100°C have been isolated (Vieille and Zeikus, 2001). It was hypothesised that amylases from hyperthermophilic organisms might be inherently heat stable to hydrolyse starches in the natural environmental (Vieille and Zeikus, 2001).

The candidate organism: *Pyrococcus furiosus* was of great interest for the development of a sterilisation TTI because of the reported heat stability of its amylases (Koch, *et al.,* 1990). The archaeon was isolated by Fiala and Stetter (1986) from shallow

thermal waters near Vulcano Island, Italy. *P. furiosus* is an obligate anaerobic, hyperthermophilic archaeon and according to its genomic sequence, *P. furiosus* contains at least five enzymes that would be predicted to have amylase-type activity and a recombinant form of an extracellular amylase has been characterized (Jorgensen, Vorgias and Antranikian, 1997). *P. furiosus* is therefore a potentially rich source of amylolytic-type enzymes, although their exact function and the precise pathway by which starch is metabolised is not clear. It should be remembered that a strong tenet through out this work was to express these amylases from the native organism and not to genetically modify it or express the amylase via a third party vector.

*P. furiosus* amylase activity has been measured over broad temperature (40 - 140°C) with an optimum activity at close to 100°C No loss of activity was detected after 6 hours of incubation at 90°C and at 120°C and about 10% of the initial activity was measured after 6 hours (Koch *et al*., 1990),. This equated to a decimal reduction time at 120°C of 6 hours ($D_{120} = 6$ h). To inactivate the enzyme completely, incubation had to be performed at 130°C for at least 1 h. Thus this material looks suitable for a sterilisation TTI.

But for successful use, the kinetics of the thermal destruction of amylase need to show sufficient heat stability for some of the active amylase structure to remain after several minutes heating at 121.1°C, characterized by the D-value i.e. partially survive an $F_0$ 3 cook. However, the amylase must also display a z-value close to **10°C,** and so represent *Clostridium botulinum* spores.


**Materials and Methods**

Work was undertaken at the University of Georgia, USA to grow *Pyrococcus furiosus* on a rich medium containing yeast extract with peptides as the primary carbon sources (Adams *et al*., 2001; Schut *et al*., 2003). Total supernatant protein was extracted via an ammonium sulphate cut. This was dialysed and freeze dried to produce a freeze-dried powder (FDP) which could be rehydrated for preparation of TTIs. TTI tubes, were made in a similar fashion to those used for pasteurisation TTIs (Tucker *et al.,* 2003).

A starch-iodine assay was used to assess amylase activity by incubating at 92°C a mixture of soluble starch, acetate buffer (see Tucker *et al*, 2006 for details). This assay was chosen for the *Pyrococcus furiosus* amylase because of the need to incubate at temperatures above 90°C for the analyses.

From a series of isothermal experiments D-values were derived. However, the log-linear relationship between these D-values and temperature to allow for the z-value to be determined could not be initially performed due to insufficient FDP. Initial isothermal trials centred on measuring the D-value at 121°C to confirm the amylase heat stability. Non-isothermal methods of obtaining D- and importantly z-value data were used (De Cordt *et al*., 1992). Sterilisation TTIs were attached to temperature probes and the temperature-time profile, T(t) recorded. After the TTI had been through the process it was assayed. Amylase activities from the TTIs and the temperatures from the probes were converted sterilisation values using the familiar Ball equation and its analogous form for TTIs (Equation 1):

$$F = \int_0^t 10^{\frac{T(t)-Tref}{z}} . dt = D_t \left( \frac{A_1}{A_2} \right) \tag{1}$$

where A1 is the initial amylase activity and A2 the remaining activity after processing

Two variables define the F-values calculated with the sterilisation TTI and with temperature sensors: $D_T$-value for reduction in amylase activity as measured with the sterilisation TTI and the z-value as calculated from measured times and temperatures. A number of matching pairs of TTIs and integrated temperature values gave pairs of calculated F-values. To obtain estimated values for $D_T$ and z the sum of the minimum absolute difference between matching paired values was selected. Two sets of experimental trials were carried out to challenge the measurement range of the TTI and thus estimate $D_T$ and z. The data sets used different product heating rates as well as different process temperatures between 121 and 131°C.

Trial 1: The first processing style used a commercial Lagarde steam-air retort. Products were packaged in plastic pouches and glass jars. Various different thermal processes were given to achieve commercial values for sterilisation. Different heating rates from the products allowed the time-temperature data to change the lethal rate accumulation. $D_T$ and z from trial 1 were used to estimate F-values for trial 2.

Trial 2: The second processing style was a bar simulator for an FMC reel and spiral cooker-cooler with cylindrical metal cans. In this system, fast axial rotation (FAR) occurred during parts of the process. This resulted in extremely efficient heat transfer. Water (0), 1 and 2% w/w starch solutions were used to produce different heating rates in the product. Two different process temperatures were used to provide data to challenge the kinetic calculations at 124 and 131°C.

For each system one sterilisation TTI was attached to the tip of a Tracksense (Ellab UK Ltd, Kings Lynn) temperature sensor at a common measuring position.


**Results**

*Measurement of $D_T$ by isothermal methods*

Due to the limited amounts of enzyme available a high concentration of FDP was used (25 mg/ml), and the TTI solution diluted (5 mg/ml) before the assay as compared to the commonly used pasteurisation TTIs. This allowed four replicates to be produced from the one sample and so four points were obtained for calculating the gradient. Figure 1 shows the plot of logarithm of activity ratio (initial activity / final activity) as a function of heating time. The $D_{121}$-value was calculated from the regression line as 22.5 minutes.

*Measurement of $D_T$ and z by non-isothermal methods*

The advantage of non-isothermal TTI calibration is that it is representative of the behaviour of foods during thermal processing. Kinetic data (i.e. D and z) were evaluated with a series of coupled equations. The parameters used to determine values for $D_{121.1}$ and z were the differences between F-values calculated from the t-T data (referred to as F(t-T)) and from the TTI data (referred to as F(TTI)). Equation (1) shows that calculations for F(t-T) require the z-value as the input kinetic parameter, whereas those for F(TTI) requires the D-value. Hence it was possible to

estimate optimal values for the $D_{121.1}$ and z.

Figure 2 show the data for the trials. The best fit-line between paired values of F(t-T) and F(TTI) was adjusted to go through the origin; this had a minimal effect on $D_{121.1}$ and z. It was likely that the minimum measurement for this sterilisation TTI did not extend much below an F-value of 3 minutes, so the lower region of the graph might be subject to a higher error. Data from trial 2 were evaluated using the same $D_{121.1} = 21.45$ minutes and z = 9.95 C° to check on consistency. It can be seen from Figure 2(b) that there was good agreement between F(t-T) and F(TTI), although the highest F-values were 30-40% different. This level of accuracy was outside of that suggested by Pflug (1987) in which he justified a 20% difference. Improvement in the accuracy will be achieved when more amylase becomes available for testing and the kinetic experiments can be conducted with replication. However, the accuracy reported here was acceptable for a novel TTI system and demonstrates the potential for amylase from *Pyrococcus furiosus* as a sterilisation TTIs.

**Discussion**

The data illustrated that an amylase from *Pyrococcus furiosus* displayed a thermal behaviour that was suitable for use as a sterilisation TTI. D-values at 121°C were measured as 22.5 minutes for isothermal calibration and 24.5 minutes for non-isothermal calibration. Non-isothermal calibration for the z-value gave 10 C°, which was the same as the *Clostridium botulinurn* value of 10 C°. Fo-values measured with the sterilisation TTI were accurate to within 1.5 $F_0$-value units of the F-values from thermocouples over most of the measurement range. The exception was for the single $F_0$-value of 28 minutes where the TTI system gave a lower value. Obtaining high accuracy at high Fo-values is not as important for process safety where the operating region is in the lower range towards Fo 3. It may be that the sterilisation TTI cannot be used to measure more than one log reduction in amylase activity at the 25 mg/ml FDP concentration. Operating ranges and further definition of accuracies need to be determined when more FDP is available.

The calibration of any measurement system is an essential requirement in order to provide confidence that the values are correct and within a defined error band (Mehauden *et al.*, 2006). Estimated errors displayed in Figures 2 were ±10% on time-temperature F-values and ±12.5% on TTI F-values. These errors were calculated from estimations of inaccuracy with the measurement systems and variability with the relative experiments. Thermocouple temperature measurements were assumed accurate to within ±0.5°C under non-isothermal conditions, which converted to about ±10% at 121.1°C. Estimated accuracies with TTI F-values were based on a change in D-value of ±3 minutes from the 24.5 minutes calculated from the non-isothermal tests. This represented the upper and lower D-value limits from the non-isothermal calculations that gave acceptable agreement between F-values from paired TTIs and probes. Further work will be needed to confirm whether this is a realistic assessment and appropriate refinements made.

F-values predicted using the calculated $D_{1211}$-value for the sterilisation TTIs were consistently within 1.5 F-value units of those from the time-temperature data for F-values in the range 3.0 to 11.0 minutes. With most in-pack thermal processes operating at around Fo-values of 6 to 12 minutes, this is an acceptable measurement range and level of accuracy. Continuous thermal processes with particulates usually operate to substantially higher Fo-values because of the uncertainty involved with their measurement. Thus, an error of ±1.5 minutes on a measured F-value in the region of

20-30 minutes would not be an issue.

**Conclusions**

A candidate sterilisation TTI has been identified and tested based on protein extracts containing *Pyrococcus furiosus* amylase. The measurement range for this sterilisation TTI allowed two objectives to be realised; namely measuring process values at 121$^{o}$C and to allow for process optimisation.

Limitations in the quantity of FDP did not make it possible to complete all the testing. Further experimental work is planned to address the following:

- The best storage conditions need to be determined for the FDP and of the prepared sterilisation TTI tubes.

- What level of amylase purification is required? This is because a reduction in activity was found when the sterilisation TTIs were stored chilled, and it thought to be throught the action of proteases in the FDP.

- What intrinsic variability should be expected for the sterilisation TTI? As TTIs will have many applications in industrial thermal processes it will be necessary to understand the absolute accuracy of F-values estimated from the TTIs (see Mehauden *et al.*, 2006).

- How to guarantee long term supply of the FDP with reproducible heat stability properties. *P. furiosus* fermentation may not be the best method to produce heat stable amylase. There are reports of the gene being expressed in bacteria such as *E. coli* or in moulds. Reports suggest that the amylase from an *E. coli* retains its heat stability but it has not been tested in the same way as for a sterilisation TTI. But this approach may not be applicable for all companies due to concerns relating to GMO's and genetic engineering.

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Vieille, C and Zeikus, G. (2001). Hyperthermophilic enzymes: Sources, uses and molecular mechanisms for thermostability. Microbiology and Molecular Biology Reviews, 65 (1), 1-43.
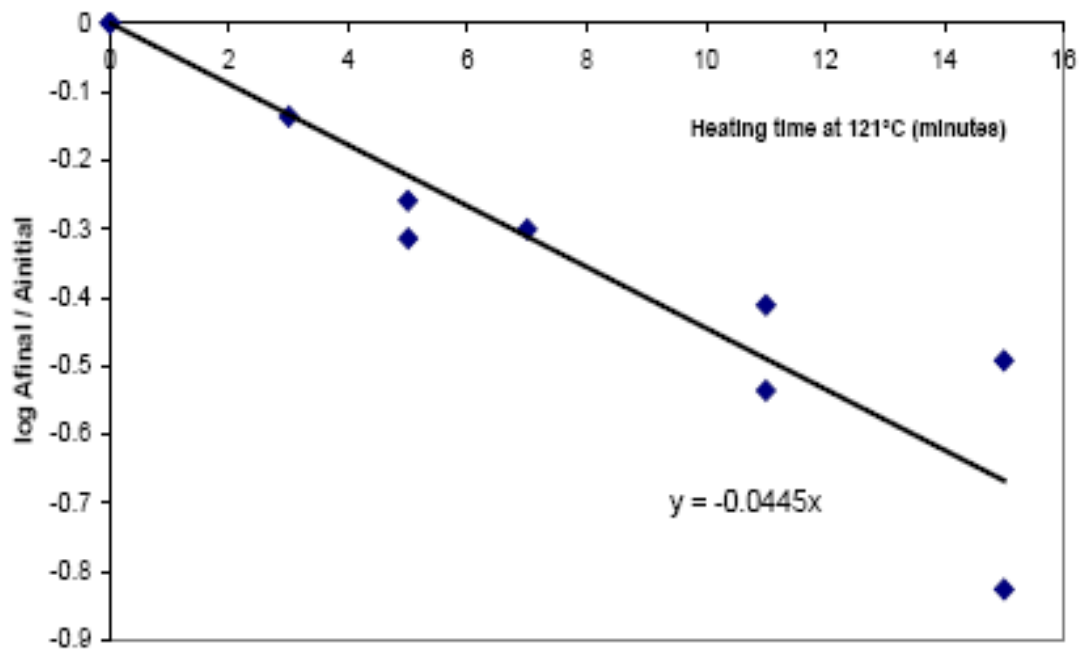
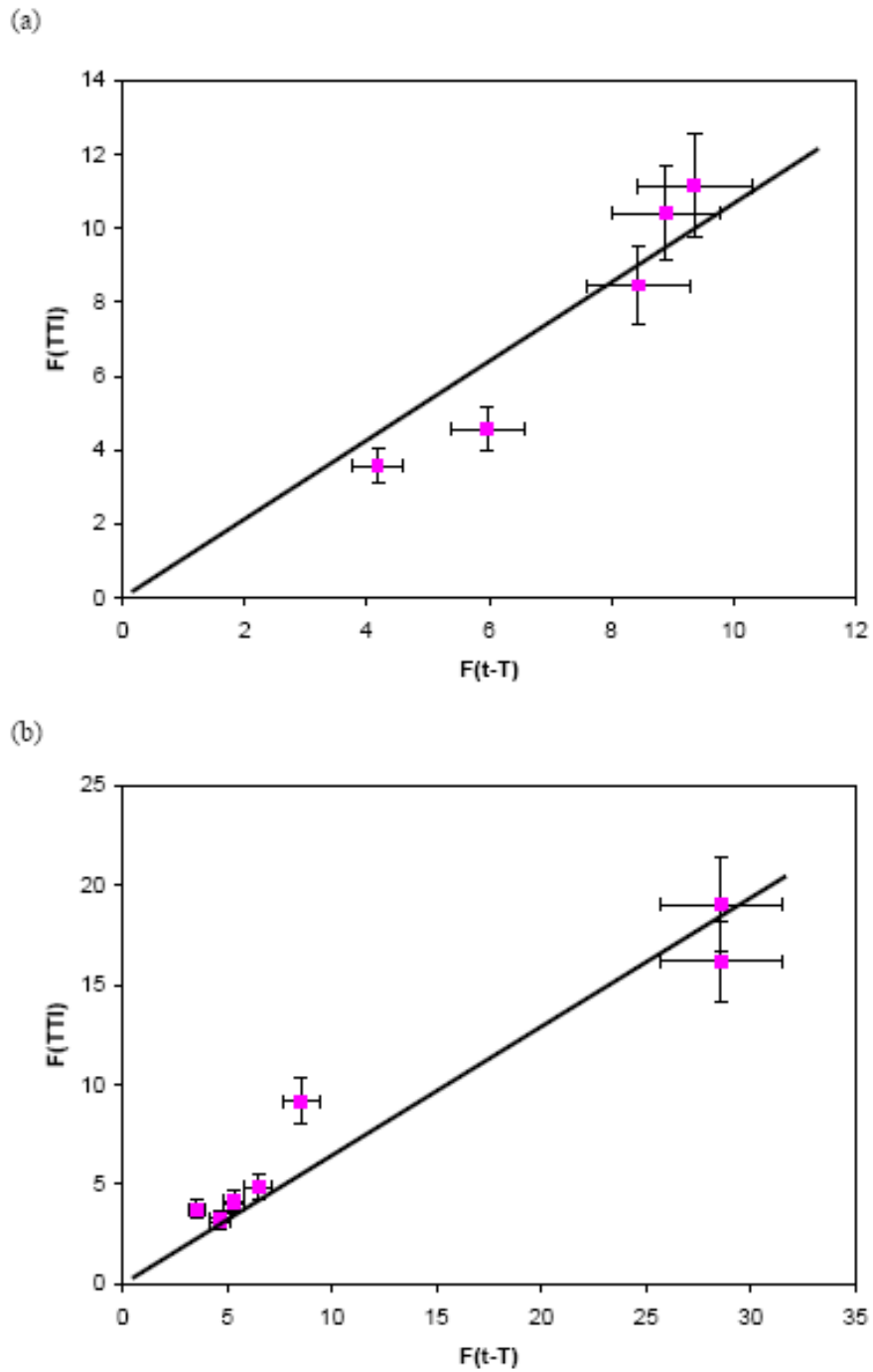Figure 1. The ratio of activity for isothermal heating at 121oC. D= 22.5 mins.

Figure 2. F(t-T) and F(TTI) graphs for trials calculated using $D_{121.1}$ of 21.4 mins. and a z of 10$^o$C. Fig. 2a pouches in a Lagarde steam/air retort, Fig. 2b. cans in a FMC reel and spiral cooker/cooler.